RECEIVED
CENTRAL FAX CENTER
SEP 16 2005

Attorney Docket No. 1059.00073 PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of: Michael Chopp, et al.

Serial No. 10/075,715 Group Art Unit: 1614

Filed: 02/13/02 Examiner: GEMBEH, Shirley V.

For: NITRIC OXIDE DONORS FOR INDUCING NEUROGENESIS

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

DECLARATION

I, Michael Chopp, being duly sworn, do hereby state that:

1. I am one of the inventors named in the above-captioned application.

2. I am skilled in the art and have worked extensively in the field of Nitric Oxide Donors for Inducing Neurogenesis.

At the request of the Examiner responsible for the above-captioned patent application enclosed herewith is data establishing that the method as claimed in the presently pending independent claims both increases levels of cGMP and causes neurogenesis. Attached are four papers, all utilizing the methodology disclosed in the above-captioned patent application, which provide the requested data. Additionally, my lab performed the following experiments.

We provide data to demonstrate that administration of sildenafil, a specific inhibitor of phosphodiesterase type 5 (PDE5), significantly amplifies neurogenesis in stroke brain of young and aged rats through increases of cyclic guanosine monophosphate (cGMP) levels.

Neuronal progenitor cells in the subventricular zone (SVZ) of the lateral ventricle and in the dentate gyrus of the hippocampus can proliferate throughout the life of the animal. To examine whether administration of sildenafil affects neural proliferation in these two areas, sildenafil at 2mg/kg or 5mg/kg dissolved in 3 ml of tap water was

administered orally to young (3 – 4 months) rats 2 hours after MCA occlusion and daily for an additional 6 days. Another group of the ischemic young rats was treated orally with sildenafil (2mg/kg) 24 hours after MCA occlusion and daily for an additional 6 days. Stroke rats were treated with the same volume of tap water as a control group. Bromodeoxyuridine (BrdU), the thymidine analog that is incorporated into the DNA of dividing cells during S-phase, was used to measure cell proliferation. Animals received daily (I.P) injections of BrdU (50mg/kg) on the day of stroke and subsequently for 14 consecutive days. Cell proliferation in the SVZ and dentate gyrus were measured in rats sacrificed 28 days after stroke.

We found that stroke rats treated with sildenafil initiated at 2 or 24 hours after stroke had significant ($p<0.05$) increases in numbers of BrdU immunoreactive cells in the dentate gyrus of both hemispheres compared with control rats (Table1). Treatment with sildenafil at a dose of 2 mg/kg (at 2 or 24 hours) significantly ($p<0.05$) increased numbers of BrdU immunoreactive cells in the ipsilateral SVZ, and the 5 mg/kg dose (at 2h) significantly ($p<0.05$) increased numbers of BrdU immunoreactive cells in the SVZ of both hemispheres compared with numbers of BrdU immunoreactive cells in control group (Table 1).

Using antibodies against βIII-tubulin (TuJ1), a marker of immature neurons, we found that treatment with sildenafil significantly increased the number of TuJ1 immunoreactive cells in the ipsilateral SVZ (Fig. 1A and H) and striatum (Fig. 1C and I) of young rats 28 days after stroke compared with stroke rats in the control group (Fig. 1B, 1C, H and I). Double immunostaining with antibodies against TuJ1 and BrdU shows that BrdU immunoreactive cells (Fig. 1E and 1G, green, arrows) were TuJ1 immunoreactive (Fig. 1E and 1F, red, arrows), indicating that these cells are newly generated neurons. Collectively, these results demonstrate that treatment of stroke with sildenafil augments neurogenesis in stroke brain of the adult rat.

Stroke is a major cause of death and disability in the elderly. Accordingly, we investigated the effects of sildenafil on neurogenesis in aged rats after stroke. Sildenafil at a dose of 3 mg/kg was administered (I.P) to aged rats (18 months, n=8) daily for 7 consecutive days starting 7 days after stroke. Using cell proliferating markers, minichromosome maintenance protein-2 (MCM-2) and Ki67, we measured the number

of proliferating progenitor cells in the SVZ 3 month after stroke. Treatment with sildenafil significantly increased the number of MCM-2 positive cells in the SVZ of aged rats (Fig. 2B and 2C) compared with aged rats treated with saline (Fig. 2A and 2C, n=8).

Many of the proliferating cells (Fig. 2E, red) were doublecortin positive (Fig.2E, green), a marker of migrating neurons, indicating that these cells are newly generated migrating neuroblasts. These data demonstrate that sildenafil can augment neurogenesis in stroke brain of aged rats.

PDE 5 enzyme is highly specific for hydrolysis of cGMP. To examine whether inhibition of PDE5 activity by sildenafil increases cGMP levels, we measured brain cGMP levels using a cGMP ELISA kit. Although brain cGMP levels were lower in aged rats than young rats, treatment of stroke with sildenafil significantly increased brain cGMP levels in both young and aged rats compared with rats in the saline group (Fig. 3). These data show that sildenafil enhances neurogenesis in stroke through increases of cGMP.

To examine whether SVZ cells of the adult rat express PDE5, we measured mRNA and protein levels of PDE5 in SVZ cells derived from the adult rat. Real time RT-PCR and Western blot analysis showed the presence of PDE5 mRNA and proteins, respectively, in SVZ cells (Fig. 4A and 4B). Double immunostaining revealed that TuJ1 (Fig. 4D and 4E) or nestin (Fig. 4G and 4H) positive cells were PDE5 immunoreactive (Fig. 4C and 4F). These data indicate that neural progenitor cells identified by nestin and neurons identified by TuJ1 express PDE5, which provides molecular bases for the effects of sildenafil on neurogenesis in the adult brain.

To investigate direct effects of sildenafil on neurogenesis, we performed *in vitro* experiments. Neural progenitor cells isolated from the SVZ of adult brain were incubated with different concentrations of sildenafil. We found that sildenafil at doses of 150 and 300 nM significantly increased the number of TuJ1 positive cells (Fig. 5A and 5C) compared with the control group (Fig. 5B and 5C). Incubation of neural progenitor cells with 8-Br-cGMP (100 μM), a cGMP analog, also significantly increased the number of TuJ1 positive cells (Fig. 5D). These data demonstrate that sildenafil enhances neurogenesis via increases of cGMP and strongly support our in vivo findings. .

of PKG. Neural progenitor cells were incubated with sildenafil (300 nnM) for 2 and 6 hours and mRNA levels of PKG were measured by quantitative real-time RT-PCR. We found that incubation of neural progenitor cells with sildenafil increased expression of type II PKG by 4 to 6 fold ($p<0.05$) (Fig. 6) but not type I PKG, suggesting that type II PKG may be involved in sildenafil induced neurogenesis.

The undersigned declares further all statements made herein of his knowledge are true and that all statements made upon information and belief are believed to be true, and further that the statements were made with the knowledge that willful and false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Date: September 15, 2005

Michael Chopp

Table 1. Density of newborn cells in the brain

Groups	SVZ		Dentate gyrus	
	Ipsilateral	Contralateral	Ipsilateral	Contralateral
Sildenafil (2mg/mg 2h)	382.91 ± 23.44*	296.22± 19.74	54.67 ± 3.99*	55.42 ± 2.10**
Sildenafil (5mg /mg 2h)	436.66 ± 32.97**	311.66± 23.79*	59.41 ± 5.26*	58.37 ± 5.38*
Sildenafil (2mg /mg 24h)	374 ± 16.07*	295.2 ± 24.54	56.97 ± 4.21*	56.16 ± 4.76*
Control	295 ± 32.69	245.86 ± 18.54	43.68 ± 2.96	42.46 ± 3.01

Density of newborn cells is presented as the mean number of BrdU-immunoreactive cells per mm^2 ± SEM. *p<0.05, vs the control group.

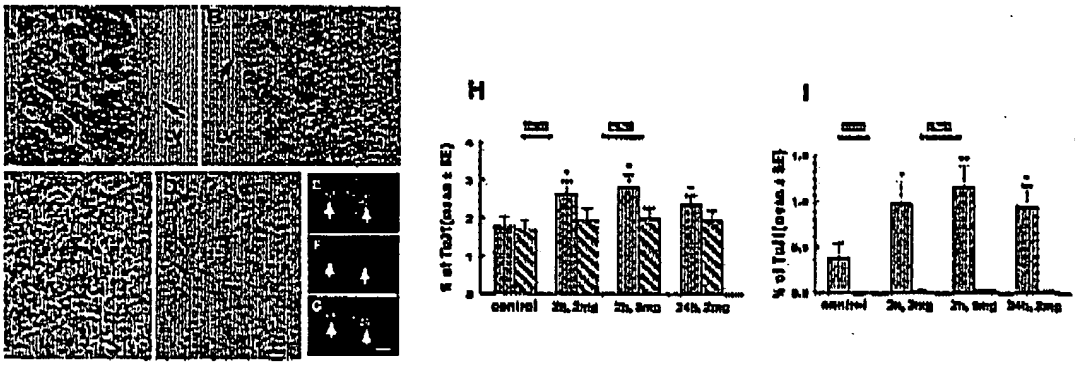


Fig 1. From a representative rat, panel A shows robust increases in numbers of TuJ1 immunoreactive cells in the ipsilateral SVZ compared with the contralateral SVZ (Panel B). Ependymal cells (arrows in Panels A and B) were not TuJ1 immunoreactive. TuJ1 immunoreactive cells exhibited cluster in the ipsilateral striatum (C) as compared to the homologous tissue in the contralateral hemisphere (D). Double immunostaining with antibodies against TuJ1 and BrdU shows that BrdU immunoreactive cells (E and G, green, arrows) were TuJ1 immunoreactive (E and F, red, arrows). Panel E is a merged image from panels F and G. Panels H and I show quantitative data of numbers of TuJ1 immunoreactive cells in the SVZ and striatum, respectively. *p<0.05, **p<0.01, and #p=0.05 versus the saline group. LV = lateral ventricle. Bars in B and G = 10 μm and in C = 20 μm .

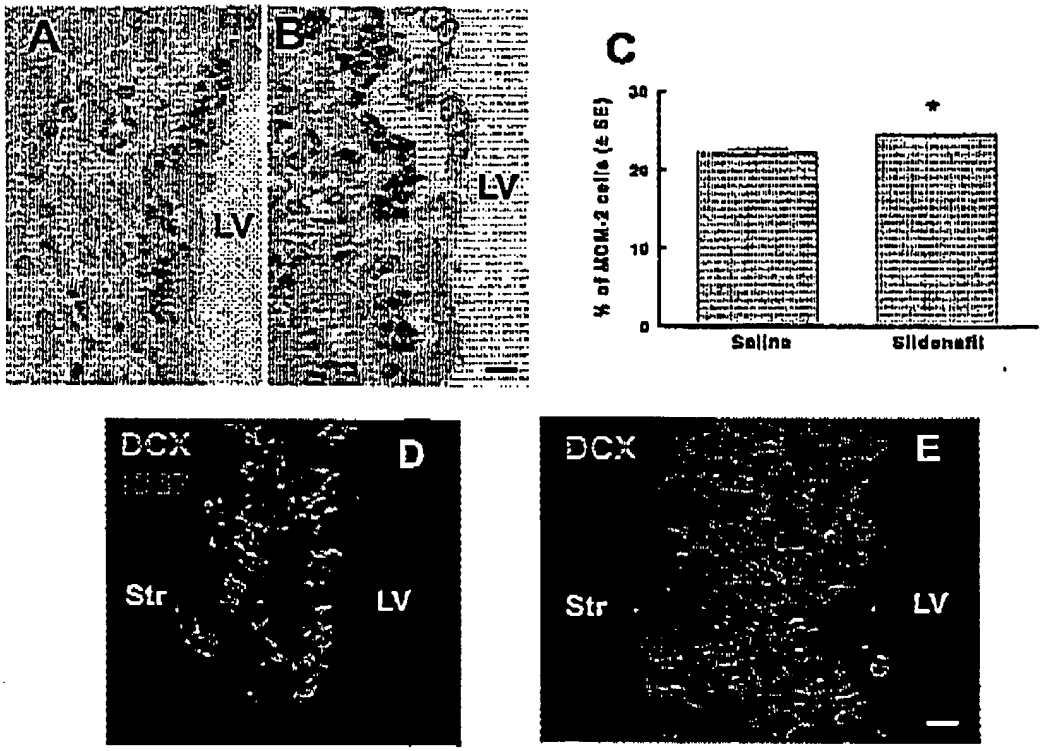


Fig. 2 Treatment of stroke with sildenafil increases neurogenesis in the SVZ of aged rats. Panels A and B show MCM-2$^+$ cells in the SVZ of representative aged rats treated with saline (A) and sildenafil (B). Panel D and E show double immunostaining for Ki67$^+$ (red) and DCX$^+$ (green) cells in the SVZ of the representative aged rats treated with saline (D) and sildenafil (E) after stroke, respectively. Panels C shows quantitative data of numbers of MCM-2$^+$ cells in the ischemic SVZ. *$p<0.05$, **$p<0.01$, and #$p=0.05$ versus the saline group. LV = lateral ventricle and Str = striatum. Bar = 10 μm.

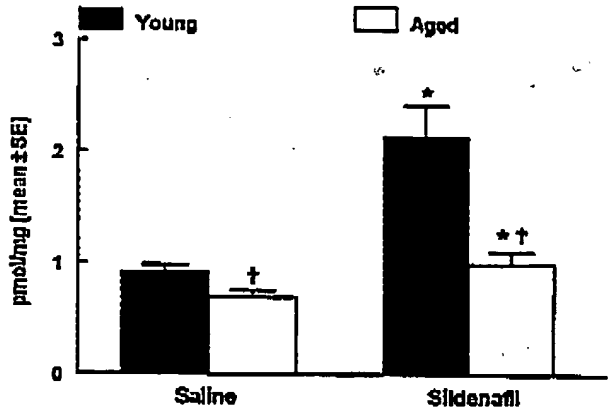
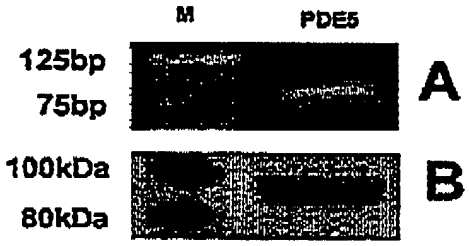


Fig 3. Treatment of stroke with sildenafil increases ipsilateral cortex cGMP Levels in young and aged rats at 7 days after MCA occlusion. *P<0.05 vs the saline treated respective groups. + P<0.05 vs respective young groups.



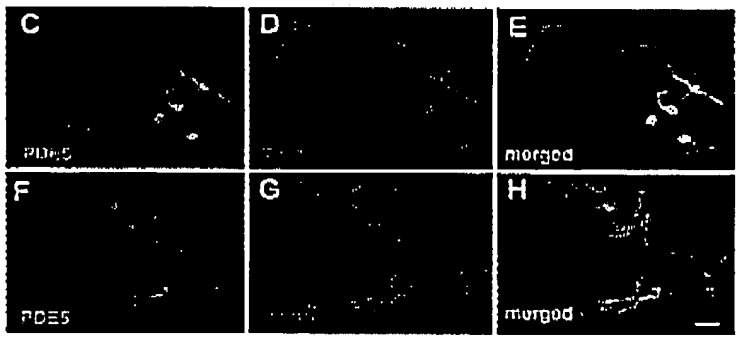


Fig. 4. Neurospheres express PDE5. Real time RT-PCR (A) and Western blot (B) analysis show the presence of PDE5 mRNA and proteins, respectively, in neurosphere (n=3). Double immunostaining revealed that PDE5 positive cells (C, E, F, H, green) were TuJ1 (D, E, red) and nestin (G, H, red). Bar = 10 μm.

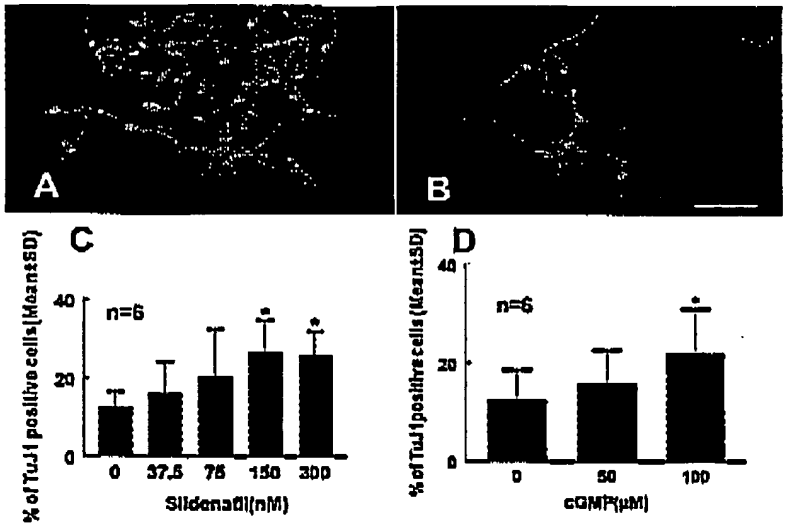


Fig. 5 Sildenafil enhances neuronal differentiation in vitro. Panels A and B show TuJ1 immunoreactive cells (red) in the Sildenafil treated and control groups, respectively. Quantitative data show the effect of Sildenafil (C) and 8-Br-cGMP (D) on the numbers of TuJ1 positive cells. *P<0.05 vs. the control group. Bar=100 μm

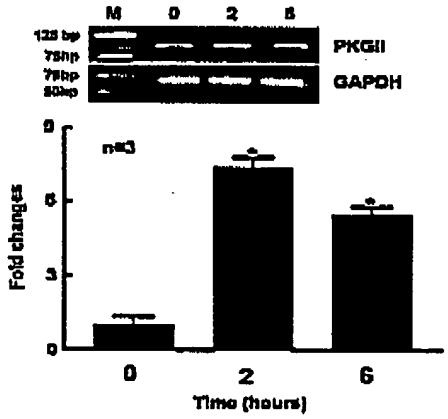


Fig. 6. Quantitative measurements of real time RT-PCR show that incubation of neural progenitor cells with sildenafil significantly increased type II PKG mRNA2 and 6 h after incubation. GAPDH was used as an internal control. *P<0.05 vs. the saline group. The zero time point is the saline group.